Final synopsis of the Ph.D. Thesis entitled

Development and validation of sensitive immunoassay methods for estimation of inflammatory marker C-reactive protein

In fulfilment of the requirements for the award of the Degree of Doctor of Philosophy in Department of Applied Zoology

By,
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1 Introduction

Human C-reactive protein (CRP) is an acute-phase protein in human blood serum; it was accidentally discovered by Tillett and Francis (1930) while working with *Streptococcus pneumoniae*. CRP is an annular (ring-shaped) protein with five identical subunits consisting of 206 amino acid residues in each subunit and belongs to the pentraxin protein family (Kindmark, 1971; Volanakis, 2001; Ansar, 2020). Based on primary observation, CRP was named after its unique characteristics to react and bind to somatic capsular polysaccharide C like a ligand; initially, it was observed in *Streptococcus pneumoniae* (Holzer *et al.* 1984), later studies revealed that the binding affinity of CRP is towards the phosphocholine and this interaction is calcium-dependent (Abernathy and Avery, 1941; Agrawal, 2005; Pathak and Agrawal, 2019). Furthermore, it was found that CRP reactivity to phosphocholine triggers the innate immune system using the C1q molecule by activating the classical complement pathway (Volanakis and Kaplan, 1974; Agrawal *et al.*, 2001). This activation leads to the release of pro-inflammatory cytokines and the activation of other immune cells, such as neutrophils and macrophages (Kushner, 2023). Ultimately, this response destroys pathogens. Hence, CRP has evolved as an acute-phase protein utilized as a systemic inflammatory marker, generally functionalized at tissue damage or site of infection (Kushner, 1982).

CRP synthesis is regulated in response to essential pro-inflammatory cytokines, especially interleukin-6 (Boras *et al.*, 2014). CRP regulation is controlled by interleukin-6, which can upregulate the CRP 10,000 times in pathogenic attacks (Kindmark, 1971; Sproston and Ashworth, 2018). The leading synthesis site of the CRP is hepatocytes. Besides hepatic cells, neurons, leukocytes and muscle cells are the CRP synthesis sites (Volanakis, 2001; Pathak and Agrawal, 2019; Ansar, 2020). CRP serum concentration is not affected by these synthesis sites. Increasing CRP concentration during inflammation is a physiological response (Pepys and Hirschfield 2003); apart from this, CRP has multiple functions associated with particular CRP level (Table 1). Serum CRP concentration indicates the association with different clinical conditions. The normal concentration of the CRP in serum is 0.8 mg/L (Kindmark,1971).

Recent studies have shown that CRP protein is a remarkable marker for cardiovascular diseases (CVD) risk management and elevation in CRP level is prognostically associated with acute myocardial infarction (Ridker *et al.*, 2001; Wilson *et al.*, 2006; Scrutinio *et al.*, 2011; Stephen *et*

al., 2015; Quispe *et al.*, 2020). In healthy individuals, CRP level indicates the CVDs risk (Ridker *et al.*, 2018). The association of CRP concentration with CVDs risk is presented in Table 2 (Minami *et al.*, 2018; Pereira *et al.*, 2018; Dong *et al.*, 2019; Saleh *et al.*, 2022). CRP concentration suggests future physiological complications such as severe ischaemic stroke and peripheral coronary disease (Paul *et al.*, 2004; Avan *et al.*, 2018).

Table 1. CRP levels associated with various clinical conditions

	Clinical conditions	CRP levels (mg/L)
1	Healthy individual	< 0.8
2	Mild systemic inflammation (viral infection or cronic condition)	1-10
3	Autoimmune disease conditions or bacterial infections	10-50
4	Pancreatitis inflammation bacterial infections or trauma	50-200
5	Sepsis or septic shock, severe burns/trauma	> 200

Considering the clinical importance of the CRP, detection procedures need to be developed, which must be sensitive, reliable, precise and reproducible. CRP quantification methods, like turbidometry and nephelometry are rapid and inexpensive, but, have low specificity and sensitivity. Hence, cannot be considered for sensitive analysis of CRP in CVDs risk management. To overcome this, rapid, precise and reproducible assays like radioimmunoassay, ELISA or chemiluminescent immunoassay must be developed.

Table 2. CRP concentration and associated cardiovascular disease risk

	Cardiovascular risk	CRP concentrations (mg/L)
1	Low risk	< 0.8
2	Moderate risk	0.8-2
3	High risk	2-3

2 Immunoassay

Immunoassay mainly involves the quantitative determination of the analytes in various biological samples using the specificity of antigen-antibody interaction. The immunoassays are classified depending on the nature of the binding protein and reaction system (competitive and noncompetitive). Radioimmunoassay (RIA) and Enzyme-linked assay (cELISA) are the most

widely used competitive immunoassays, direct ELISA, indirect ELISA, sandwich ELISA and chemiluminescent are noncompetitive immunoassay methods. The immunodiagnostic methods that employ a radioactive nuclide are called radioassays , where, usually, ^{125}I is used to determine the presence and quantity of a specific substance of interest. Similarly, in enzyme-linked assays, enzymes such as horse radish peroxidase and alkaline phosphatase have been used for detection.

2.1 Radioimmunoassay

Radioimmunoassay, a pioneer in sensitive immunoassay method was invented by Yalow and Berson in 1960. For the past decades, the relavance of the RIA has remained unchanged. RIA is a 'saturated assay' called a 'limited reagent' or 'excess analyte' assay. In saturation assays, the active reagent, antibody, is used in a much lower concentration than the analyte. The assay is based on a competitive reaction between an analyte of interest and a labelled identical analyte. The RIA principle is based on the competition of analyte A (antigen) and a ^{125}I labelled analyte A* (identical to antigen) to a specific antibody B to form the complex AB or A*B. Here, the analyte A and labelled A* are identical; hence compete for the limited antibody B provided in the reaction mixture. Due to the limited antibody B, as the concentration of the sample analytes increases, the chances of labelled analytes binding to antibodies decreases substantially. Hence, a signal in the presence of a high sample antigen decreases and a reference curve is produced based on the radiation activity associated with the labelled antigen. This reference curve is further utilized for analyte quantification from the sample.

Radiolabeling to Protein

The RIA method measures trace protein concentrations from complex biological samples (Yalow and Berson 1960). In RIA, iodine radioisotope (^{125}I), which has a half-life of nearly 60 days, is labelled to the analyte's amino acid residues tyrosine/histidine . The process of ^{125}I labelling to analyte/antibody is known as radioiodination. The labelled molecules are used as a radiolabeled tracer (Greenwood *et al.*, 1963). Various methods have been described for the radioiodination of the protein, but the Chloramine-T method has been widely accepted in various published iodination procedures (Youfeng *et al.*, 1982; Bhalla *et al.*, 1997; Bailey 2002; Rasmi *et al.*, 2021; Manupriya *et al.*, 2023) Simplicity and reproducibility make this method more popular than

others. Standardization of the iodination procedure includes the purified protein's radiolabeling, purification and validation.

Separation system for immunoassay

Separation systems are of two types: liquid phase and solid phase separation systems. In the liquid phase, the reaction immune complex is separated from free non-reacted reagents by applying the 2^{nd} antibody. However, it's expensive because the 2^{nd} antibody adds one more step to the assay procedure. On the other hand, in solid-phase separation, antigen-specific antibodies are directly coupled with magnetizable cellulose particles (mag-cell parts), resulting in the same sensitivity and accuracy. A solid separation system based on antigen-specific antibodies coupled with mag-cell parts has been a well-optimized coupling method (Kadwad *et al.*, 1996, Rasmi *et al.*, 2014, Manupriya *et al.*, 2023).

2.2 Enzyme-linked immunosorbent assay (ELISA)

ELISA is one of the most widely used biochemical methods in laboratory analysis and diagnostics, invented by Engvall and Perlmann (1974). Analytes such as peptides, proteins, antibodies and hormones can be detected selectively and quantified in low concentrations among many other substances. In the basic principle of ELISA, enzyme molecules are conjugated to secondary antibodies, which bind to the primary antigen-antibody complex. When the appropriate substrate is added, the enzyme catalyzes the production of coloured end-products, which can be visualized and quantitated. The enzyme most often used is alkaline phosphate (AP) or horseradish peroxidase (HRP) (Wisdom, 2005). There are four types of ELISA: indirect, competitive, sandwich and direct.

In indirect ELISA, a primary antibody is added to the analyte-coated microtiter plate. The free primary antibody is washed out and treated with an enzyme-conjugated secondary antibody that binds to the primary antibody and a specific substrate will be added. The enzyme forms a coloured product which is measured using a microtiter plate reader. In the direct ELISA procedure, an analyte-coated microtiter plate was added with a primary antibody conjugated to HRP. After the wash, the added specific substrate develops colour which is measured.

The following objectives were set for the work to develop and validate RIA and ELISA (competitive, direct and indirect) for CRP to provide a high-degree assay with adequate sensitivity

and specificity necessary to quantify the CRP in human serum samples.

1. Production of polyclonal antibody against C-reactive protein.
2. Development and validation of an indigenous RIA ^{125}I-based method for analysis of C-reactive protein in human serum. Clinical validation of the developed assay system.
3. Development and validation of an indigenous direct and indirect ELISA method for analysis of C-reactive protein in human serum. Clinical validation of the developed assay system.
4. Development and validation of an indigenous competitive ELISA for analysis of C-reactive protein in human serum. Clinical validation of the developed assay system.

The proposed thesis comprises of six chapters: introduction, literature review, development and validation of the RIA and direct, indirect and competitive ELISA for CRP and summary.
Chapter 1: This Chapter highlights the importance of the CRP, clinical analysis and the present profiling of indigenous methods in CRP quantifications.
Chapter 2: The literature review includes information on the CRP structure, biosynthesis and function in the human physiological system. In this Chapter, the physiological importance of CRP is also discussed. Chapter also provides at the end scope of the of the proposed work.
Chapter 3: The development and validation of CRP-RIA is described in two sections (I and II). Features of RIA and ELISA are given breafely. Section I contains the reagent preparation, including the coupling of CRP antibodies to the mag-cell parts, which was carried out using the CDI activation method.

1. Before coupling, characterization of the obtained Mag-cell parts was carried out using scanning electron microscopy and the average particle size was 2.5±0.5 µm, with size variation of minimum and maximum 2 and 2.8 µm, respectively.
2. In Fourier Transform Infrared Spectroscopy (FTIR) spectra, various functional groups were detected. The peaks were observed at 559.35 and 455.20 cm^{-1} that corresponds to the magnetite state vibration of the Fe-O bond. The peaks 3327.20, 2889.36, 1618.27, 1344.38 and 1004.91 cm^{-1} are the characteristic peaks of cellulose in the solution. The peak 3327.20 cm^{-1} represents –OH stretching observed due to the presence of deionized

water. All other peaks 2889.36, 1618.27, 1344.38 and 1004.91 cm^{-1} symbolize the -CH stretching, -OH bending, -CH_2 wagging and C-OH stretching in a plane at C-6, respectively, with low intensities as iron particles are immobilized on the surface of cellulose.

3. X-ray Diffractometer analysis was also carried out. The X-ray diffractogram of magnetizable cellulose particles has mainly five points of 2 θ degree reflections at 30.02°, 35.44°, 43.10°, 57.06° and 62.46° with the crystalline structure ranging from 1.48 to 3.96 %. The shape of the peak was observed to be wider than the regular Fe_3O_4 XRD pattern, which indicates the coating of cellulose molecules onto the magnetite nanoparticles.

Another critical reagent is a CRP tracer prepared by a ^{125}I isotope labelling to a highly purified CRP using the chloramine -T method (Greenwood *et al.*, 1963). Using this method, the radioiodination yield of CRP was 86.6 ± 3% with a specific activity of 17.9 µCi/ µg. The purified tracer had 98 ± 2% radiochemical purity and tracer stability was two months.

In section II, CRP-RIA development and validation is presented, including optimizing assay conditions, reagent volume verification, the effect of the reagent dilutions and the solid phase separation. Assay optimization was carried out by standardizing the standards plot with three different standards as follows.

1. Regular CRP-RIA assay range 0-6400 ng/mL
2. Cardiovascular disease CRP-RIA range 0-4800 ng/mL
3. Sensitive CRP-RIA 0-200 ng/mL

Prepared standards were compared and calibrated with the WHO international standard material for calibration. Analytical and clinical validation of the optimized CRP-RIA method was done using parameters like sensitivity, inter-assay, intra-assay, precision, recovery, linearity, assay variability and assay drift. Developed assays were utilized for the sample analysis and the results were compared with a commercial CRP ELISA kit.

Following are some features of the developed assay.

1. Standards for CRP were prepared using human-purified CRP. Three standards of different ranges were prepared in CRP-free bovine serum. Among all standards, the regular range covered the clinical condition for acute inflammation: 0, 25, 50, 100, 200, 400, 800,

1600, 3200 and 6400 ng/mL. The sensitive (Low-CRP value) range was covered in the 0, 6.25, 12.5, 25, 50, 100 and 200 ng/mL. A special standard for cardiovascular disease risk management ranged from 0, 150, 300, 600, 1200, 2400 and 4800 ng/mL. These established ranges cover the extensive range of the CRP concentration, as shown in Tables 1 and 2. These prepared standards were calibrated with the WHO international CRP standards obtained from the National Institute for Biological Standards and Control (NIBSC) reference CRP standards.

2. Controls were assayed with standards, in the assay to verify the assay linearity and confirmation of the assay system. Hence, three controls were prepared: 50, 200 and 800 ng/mL from pooled CRP-containing serum. In a pair of controls, 50 ng/mL was used for all assays for low-value standards and high-value 800 ng/mL was used similarly; in the sensitive assay, 200 ng/mL was used as the high-value control.

3. The assay incubation time was optimized by testing one, two, five hours and overnight; based on the results, 1 hour was fixed as incubation time. The assay was performed at Room Temperature (RT), 37℃, and 4℃ and RT was set as the optimum temperature condition.

4. The optimized CRP-RIA procedure: Tubes were added with 100 µL of 1:200 (regular and cardiovascular standards range) or 1:600 (sensitive standard range) diluted Anti-CRP ab-mag-cell parts, the 100 µL of 1:9 diluted CRP tracer, 50 µL of CRP-free serum and 100 µL of CRP standards 0-6400 or 0-4800 and 0-200 ng/mL or sample or control C-1 and C-2. Vortexed the tubes thoroughly and covered the tubes with aluminium foil before placing the rack at RT for 1 hour. After incubation, 1 mL of assay buffer (0.05 M phosphate buffer pH 7.4+0.2% BSA+0.1% Tween 20) was added to the magnetic rack and left for 20 minutes. The supernatant was discarded. Reading was taken on a multi-well gamma counter for a minute and recorded as counts per minute (CPM). The graph was constructed on a logit-log graph and a typical standard curve for CRP was plotted by %B/B0 on the Y-axis and CRP standards concentrations were obtained on the X-axis. Standard plot was further used for unknown sample analysis.

5. The sensitivity for the present assay system was analyzed by finding the LD90 in the

graph. Sample dilution linearity and parallelism analysis were carried out for CRP-RIA by spiking the sample/standards and diluting to two-fold, four-fold, eight-fold and sixteen-fold. Similarly, a parallelism study was carried out in the developed CRP RIA assay system. To measure recovery, known CRP concentrations were added to serum samples to prepare spiked serum samples and analyzed for recovery values.

6. Inter-assay variation was calculated by consecutive discrete concentrations of the same sample/ control in the discrete repeated assay. The Intra-assay variation was evaluated by analyzing multiple replicates of the same samples or controls run in a single assay; calculated assay variation results were < 10.8 %.

7. Blood sample collection and analysis were done for the local population, which is a population-based study from Dakshin Kannada district, Karnataka State, India. Collected blood samples were centrifugated at 1400g and serum aliquots were stored at -80°C. Approximately 5-10 ml blood samples were collected with informed written consent, followed by a detailed interview about their health issues and family background. Ethical clearance for human blood collection was obtained from the Institutional Human Ethical Committee, Mangalore University, Karnataka, India (Ethical clearance- No. MU-IHEC-2020-1, dated 21.8.2020).

8. For assay precision, two control values were established, ranging from 47 to 53 (control 1) and 785 to 815 (control 2) and one more control was used for the sensitive assay 200 $\pm$10 (control 3) and expressed as 50$\pm$3 and 800$\pm$15 for control 1 and control 2. Sample recovery ranged from 90% to 109 % in assay validation parameter studies. The intra-assay variation was 2.6 to 8.2 %, whereas the inter-assay observed was 4.5 to 7.4 % for sample 1 and sample 2, respectively. The dilution linearity and recovery ranged between 88-104 % and 92 -120 %, respectively (Table 3).

The concentration of CRP in human serum samples ranged from 75 to 160000 ng/mL. CRP-RIA was compared to sample concentrations derived from commercially available CRP ELISA kits. Consistency in the standards curve were calculated and obtained results suggest that establised assay standards are optimized for the CRP-RIA assay for clinical CRP estimation.

Table 3. Analytical performance characteristics of developed kit formulations for CRP-RIA

SL No.	Parameters	CRP- RIA
1	Standard/Sample volume	100 µL
2	Standard range	6.25-200, 150-4800, 25-6400 ng/mL
3	Sensitivity	2 ng/mL, 12 ng/mL
4	Incubation	1 h at RT
5	Precision Intra-assay variations Inter-assay variations	 8.2 % 7.4%
6	Recovery	90–114%
7	Dilution linearity	91-109%
8	Normal range	Below 800 ng/L or 0.8 mg/L
9	Stability of reagents Tracer Standards Control Solid Phase	 60 days 1 Year 2 Years 1 Years

Chapter 4: This Chapter ELISA for CRP is also classified into two sections (I and II), The development of indirect and direct ELISA for CRP. This Chapter explains reagent preparation, the CRP standards and the validation of the developed assay.

In section I, CRP indirect ELISA development is presented with standard preparation and polyclonal antibody production against CRP. Optimization of secondary anti-rabbit antibody conjugated with HRP was carried out for measurement. The assay was validated with assay parameters like specificity, precision, intra-assay, inter-assay, linearity and sample analysis (unknown concentrations of CRP). In addition, the developed kit was compared with the commercial CRP kit.

Production and characterization of polyclonal antibodies against CRP

In the developed indirect ELISA method for CRP, an essential component, primary antibody was raised in New Zealand rabbits for assay development. Production of polyclonal antibodies

against human CRP was done in rabbits. Two rabbits were injected with the CRP intramuscularly with Freund's complete and incomplete adjuvant and control rabbits were injected with saline. Standard antibody immunization protocol was followed to raise effective and reactive antibodies for CRP.

The anti-CRP rabbit polyclonal antibodies (RpAb-CRP) were verified using ELISA. The ELISA plate was coated with 100 ng/mL of CRP dissolved 0.2 M bicarbonate buffer pH 9.2 at 4^{o}C overnight. After this, the plate was washed and blocked with 2% BSA for 3 hours and treated with 100 µL of diluted serum (1:20 in buffer) for 12 hours at 4^{o}C. The plate was washed twice, and plates was added with 100 µL of 1:5000 diluted secondary antibodies (procured from Sigma) and incubated for 2 hours at RT. The plate was washed three times, followed by adding 100 µL of TMB substrate and kept in dark for 10 minutes to develop a blue colour. The reaction was stopped with stop solution (the blue colour turns yellow) and absorption was measured at 450 nm. The developed colour confirmed the presence of polyclonal antibodies against human CRP in the rabbit serum.

Dot ELISA and western blot were performed to determine the specificity and reactivity of the produced polyclonal antibody. In Dot ELISA analysis, collected rabbit serum was found to be reactive to the CRP. Western immunoblot also showed a band at ≈25 kDa (CRP) when treated with immunized rabbit serum. Thus, these results suggets that the antibodies produced are against CRP in rabbits with optimum reactivity.

Optimization of conditions for the indirect assay procedure

Parameters for the CRP coating were tested using different coating buffers (trizma, phosphate and bicarbonates) and bicarbonate buffer was finalized as the coating buffer. The temperature parameter 4°C, 37°C and RT was tested and based on the results it was found that RT or 37°C can be used as incubation temperature. The incubation period was tested for 2, 3, 5 and 12 hrs. followed by indirect ELISA protocol by coating 100 µL of CRP standards (0 to 200 ng/mL) and 3 hours of coating time was selected based on the results.

Optimized assay procedure for indirect ELISA for CRP

In an ELISA plate, the standards for CRP 0, 6.25, 12.5, 25, 50, 100 and 200 ng/mL which were prepared in 0.1 M bicarbonate buffer (pH 9.2) were added and incubated for 3 hrs., wells were washed thrice. Coated plates were blocked for 1 hour using a blocking reagent consisting of

2 % BSA followed by washing. RpAb-CRP containig serum was incubated for 2 hours at RT followed by washing the plate three times. Primary antibody-coated plate was treated with 100 µL of secondary antibody (1:5000) for 30 minutes and the plate was washed with a wash buffer three times. TMB substrate of 50 µL was added to the treated plate and incubated in the dark for 10 minutes and 100 µL of stop solution was added to wells and the plate was measured for absorption using a microtiter plate reader at 450 nm. The standards plot was obtained from the OD on the Y-axis with responses to CRP standards concentrations on the X-axis. The graph showed that the optical density at 450 nm at the X-axis was proportional to the concentration of CRP on the Y-axis. The concentration of CRP standards was estimated from the standard graph and a typical standard curve showed the highest OD at the standard concentration of 200 ng/mL of CRP. The statistic of the CRP standard curve was calculated and the values are presented in the equation of y = 0.119x + 0.187 R^2 = 0.9735. A standard curve was obtained and used to measure CRP concentrations in samples.

Quality control parameters like recovery were in the range of 93-110 %. Intra-assay variation observed for all the tested samples ranged between 7–10 %. Similarly, the inter-assay variation was 5–10 % in all the tested samples. Along with this, the precision was tested using the % CV of the samples and standards altogether and obtained variation showed linearity and would was in permisible limit. The sensitivity of the assay was calculated and found to be 0.38 ng/mL and standards were stable for 6 months at -20°C.

Section II, the development and validation of direct assay for CRP is explained. Section also includes conjugation of HRP to anti-CRP polyclonal antibodies for detection (HRP-pAb-CRP), design of assay standards, preparation of blocking reagent and standardization of assay conditions. The optimized protocol was validated using quality control parameters like recovery, linearity, intra-assay, inter-assays, etc.

Conjugation of HRP with anti-CRP polyclonal antibody

HRP conjugation was carried out using the sodium periodate oxidation method, where 1mg/mL CRP-pAb was conjugated with 10mg/mL oxidized HRP using freshly prepared $NaIO_4$ (oxidizing agent). The conjugation reaction was allowed for 2 hours in the dark at RT. The conjugation reaction was stabilized using $NaBH_4$ and this reaction mixture was dialyzed overnight. The reaction mixture was added to the column filled with Sephadex-200; the column was run with

a 0.1M carbonate buffer as an eluting solution. Saturated $(NH_4)SO_4$ was added to the reaction mixture and incubated at 4°C for 30 minutes, then purified fractions were characterized. The dialysis reaction mixture was added with BSA (20mg/mL). Finally, an equal volume of glycerol was added to the purified fraction and stored at -20^{o}C. HRP conjugated anti-CRP antibody (CRP-pAb-HRP) diluted for the 1:40 was selected (based on the checkerboard method results) as working dilutions against the standard range of 3.12 to 200 ng/mL to optimize the direct ELISA.

The direct ELISA assay procedure

The microtiter plate was coated with the 100 µL of CRP standards of 3.12-100 ng/mL prepared in 0.1 M bicarbonate buffer (pH 9.2) at RT for 2 hrs. The coated plate was blocked with a blocking solution prepared in phosphate buffer saline (PBS) for 30 minutes and washed 3 times. The blocked plate was added with 100 µL CRP-pAb-HRP (1:40) and incubated at RT, followed by a three time washes. Washed plates were treated with 50 µL of TMB substrate with detector antibodies and immediately kept in the dark for 10 minutes. The reaction was stopped by adding 100 µL of 0.2 N sulphuric acid and absorption was measured using a microtiter plate reader at 450 nm. A standard graph was plotted for the CRP concentration on the X-axis and OD at 450 nm on the Y-axis the equation of y = 0.0185x + 0.322, R^2 = 0.985, the unknown CRP serum samples were measured using the obtined standard curve. The assay's sensitivity was statistically calculated and found to be 0.34 ng/ml and in-house standards were calibrated with WHO international standards and results suggest that in-house prepared standards were found to be linear (correlation of 99.65%) with WHO standards.

Validation of the direct ELISA

Analytical parameters (Quality control), such as recovery, were in the 96-106 % range. Intra-assay variation observed for all the tested samples ranged 5.09 %. Similarly, the inter-assay variation was 6.8 % in all the tested samples. Assay parallism was carried out using serial serum dilutions (1:2,1:4,1:8 and 1:16) and found to be 95.7-105.1%. Along with this, the precision was tested using the % CV of the samples and standards together and variation was found to be within the permissible limit. The sensitivity of the assay standards was found to be 0.34 ng/mL.

Chapter 5: CRP cELISA was developed by applying the CRP antibodies coupled with mag-

cell-parts. The development of CRP cELISA is a clinical tool for quantifying human blood CRP concentration. The development of CRP-cELISA in this Chapter explains reagent preparation, the standards of CRP and the validation of the developed assay. cELISA development included fabricating the magnetic rack for the separation system, reagents like HRP-CRP detector and CRP antibodies coupled with mag-cell parts.

The essential apparatus, magnetic rack was fabricated using Neodymium magnetic disks (3x8mm). The base of the magnetic rack was constructed from plastic (non-magnetic material). Rack was designed as ELISA microtiter plate must mount and demount easily, which was comparable in size to the ELISA plate. A magnetic rack effectively separates mag-cell parts attached for the detection.

Conjugation of the HRP to the purified CRP

The most commonly used enzyme for detection is HRP. HRP was conjugated to CRP and used as a tracer, 5 mg/mL HRP was diluted in sodium bicarbonate and treated with 1-Fluoro-2,4-dinitrobenzene (FDNB). 2 mg/mL FDNB-treated HRP was oxidized using the 0.1 M sodium periodate and incubated in dark for 30 minutes. The oxidation reaction was terminated using 1mL of the 0.16 M ethylene glycol solution and incubated for 1 hour. Then, the reaction solution was dialyzed against 3 litres of 0.01M sodium carbonate (pH 9.5) with multiple changes. Oxidized HRP was allowed to react with 0.5 mg of CRP diluted in 0.2 sodium carbonate buffer pH 9.5. This conjugation reaction was stabilized using a reducing agent, a freshly prepared sodium borohydride ($NaBH_4$) dissolved in 0.5 mL of deionized water. The conjugate mixture was dialyzed against phosphate buffer saline pH 7.5 overnight at 4℃. The dialysis mixture was separated and purified using a column containing the Sephadex G-150 and eluted tubes were characterized by evaluating the fractions.

Fraction characterization to select the CRP-HRP fraction

In a microtiter plate, 50 µL of the diluted pooled fractions were added to each well 1-40. Then, 50 µL of TMB was added to the fraction-containing wells and incubated for 10 minutes at dark and the substrate reaction was stopped using 0.2 N H_2SO_4 and absorbance was measured at 450 nm using a microtiter plate reader. Based on the results, the fraction containing CRP-HRP fraction was selected and diluted as 100 µL, which must give approximately 2.3 OD that was selected as the dilution factor. So, the CRP-HRP tracer was suspended at 1mL per vial

and stored at -20℃ for further use and the stored tracer was stable for six months at freezing conditions.

Antibody working dilution was optimized by diluting the anti-CRP antibodies coupled with mag-cell parts with various dilutions (1:5 to 1:2560) in cELISA protocol. The performed assay protocol included 50 µL of solid phase and 50 µL of the prepared tracer to the microtiter plate. This reaction mixture was added with free serum 25 µL and incubated overnight. After incubation, 200 µL wash buffer was added to each well and kept on a magnetic rack for 20 minutes and the reaction mixture was gently decanted. 50 µL TMB substrate was added to the wells and kept in dark condition; the enzyme-substrate reaction was stopped with 50 µL of stop solutions and absorption (OD) was measured at 450 nm. Hence, 1:50 anti-CRP antibodies coupled with mag-cell-parts dilution were fixed as working dilution.

Optimization of the assay contions

Assay conditions like assay incubation time and temperature were selected for the assay optimization. Two hour incubation was selected for equalibrium reaction and RT was suitable for the incubation temperature with continious shaking.

Optimized CRP-cELISA procedure

In a blocked ELISA plate, 25 µL Sample/control/standard concentrations ranging from 0-100 ng/mL (100, 50, 25, 12.5, 6.25 and 3.12 ng/mL), 25 µL of bovine-free serum, 25 µL of the anti-CRP ab coupled to mag-cell parts and 50 µL of HRP-CRP tracer were added. The reaction was allowed for 2 hours at 25℃ by shaking. After incubation, separation was carried out by adding 200 µL wash buffer and kept for separation over the magnetic rack for 10 minutes; the supernatant was decanted carefully and the plate was removed from the magnetic rack. Then, 50 µL of TMB was added to the wells and kept in the dark for 10 minutes. The reaction was stopped using 0.2 N H_2SO_4 stop solution, where the blue colour turned yellow. The obtained reaction plate was measured for OD at 450 nm, OD was plotted at the Y-axis and CRP standard concentrations were plotted at the X-axis. The calculation used the % B/B0 formula, where B is the standard concentration and B0 is the zero standard concentration. An unknown sample value can be calculated using the graph.

Validation of the CRP-cELISA

Analytical parameters (Quality control) are tested for parameters like assay recovery, sample

dilution effect, assay senstivity and precision and the features are presented in Table 4.

Table 4. Analytical performance characteristics of developed kit for CRP-cELISA

SL No.	Parameters	CRP- cELISA
1	Standard/Sample volume	25 µL
2	Standard range	3.12-100 ng/mL
3	Sensitivity	4 ng/mL
4	Incubation	2 h at RT with Shaking
5	Precision	
	Intra-assay variations	6.2 %
	Inter-assay variations	7.2%
6	Recovery	94–105%
7	Dilution linearity	96-108%
8	Normal range	Below 800 ng/L or 0.8 mg/L
9	Stability of reagents	
	Tracer	6 months
	Standards	1 Year
	Anti-CRP Ab-mag-cell-part	1 Years

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Publications

1. Ghodke Tanhaji Sandu , Kadwad, V, Paradkar, S, Mirapurkar, S, Karunakara, N and K Bhasker Shenoy, (2025). Development of radioimmunoassay for human C-reactive protein using magnetizable cellulose particles. Journal of Radioanalytical and Nuclear Chemistry, 334(1), 551-564.

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Conference presentations

1. Ghodke Tanhaji Sandu, Vijay Kadwad, N. Karunakara, K. Bhasker Shenoy (2025).“Two novel, rapid and simple Enzyme-linked immunoassay for C-reactive protein; A cardiovascular marker” Baltic-Polish School of Immunology (BPSI 2025) organized by Baltic and Polish Immunological Societies at the Vilnius University Life Sciences Centre in Vilnius, Lithuania, (12-13 May, 2025).

2. 2.Ghodke Tanhaji Sandu , Vijay Kadwad, Shalaka Paradkar, N. Karunakara, K Bhasker Shenoy (2025). “Radiolabelling of anti-C-reactive protein antibodies for Immunoradiometric assay development” 35^{th} IARP National Conference (IARPNC-2025), organized

by the Centre for Advanced Research in Environmental Radioactivity (CARER) and Centre for Application of Radioisotopes and Radiation Technology (CARRT), Mangalore University, Mangalagangothri, Mangalore (29-31 January 2025).

3. Ghodke Tanhaji Sandu, Vijay Kadwad, Shalaka Paradkar, N. Karunakara, K. Bhasker Shenoy (2024). "C-reactive protein: A cardiovascular marker, Quantify using Radioimmunoassay" 67^{th} Annual Radiobioassay and Radiochemical Measurements Conference (RRMC 2024) organized by Purdue University, and the Dallara Indycar factory in speedway, West Lafayette and speedway, IN, United States of America, (21-25 October, 2024).

4. Ghodke Tanhaji Sandu, Vijay Kadwad, Shalaka Paradkar, N. Karunakara, K. Bhasker Shenoy (2024). "Radioimmunoassay development for Human C-reactive protein" 3^{rd} DAE-BRNS Symposium on Current Trends In Analytical Chemistry (CTAC–2023) organized by Board of Research in Nuclear Sciences and Department of Atomic Energy (DAE), DAE Convention Centre, Anushaktinagar, Mumbai (06-09 March, 2024).

5. Ghodke Tanhaji Sandu, Vijay Kadwad, Shalaka Paradkar, N. Karunakara, K. Bhasker Shenoy (2023). "Application of the magnetizable cellulose particle in development of CRP radioimmunoassay" 16^{th} Biennial DAE-BRNS Symposium on NUCLEAR AND RADIOCHEMISTRY (NUCAR-2023), organized by Department of Atomic Energy (DAE), Government of India, DAE Convention Centre, Anushaktinagar, Mumbai (01-05 May, 2023). **Best Oral Presentation award**

6. Ghodke Tanhaji Sandu, Vijay Kadwad, Shalaka Paradkar, N. Karunakara, K. Bhasker Shenoy (2023). "I-125 labeling to C-reactive protein to development of radioimmunoassay" First International Conference on Radiation Awareness and Detection in Natural Environment (RADNET-23), organized by Dolphin (PG) Institute of Biomedical and Natural Sciences Dehradun, India (02-04 March, 2023). **Best poster presentation award**

7. Ghodke Tanhaji Sandu, Shalaka Paradkar, Vijay Kadwad, Karunakara N, K. Bhasker Shenoy (2023). "Development of the high sensitive (hs) Radioimmunoassay (RIA) for C-reactive protein by using magnetizable particle" 23^{rd} National Symposium on Radiation Physics (NSRP) organized by the Department of Studies in Physics, University of Mysore and the Indian Society for Radiation Physics (ISRP) Mysore, Karnataka, India (19-21

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9. Ghodke Tanhaji Sandu, B. R. Manupriya, V. Kadwad, S. N. Paradkar, G. Rani, N. Karunakara, K. Bhasker Shenoy (2022). "Optimization of the Radioimmunoassay (RIA) procedure for C-reactive protein" 15^{th}Biennial DAE-BRNS Symposium on NUCLEAR AND RADIOCHEMISTRY (NUCAR-2021), International conference, organized by Department of Atomic Energy (DAE), Government of India, DAE Convention Centre, Anushaktinagar, Mumbai (22-26 February, 2022).

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